09/155921

PCT/EP97/01741

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

96105679.3

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 96105679.3
Demande n°:

Anmeldetag:
Date of filing: 11/04/96
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
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Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Process for the production of plants with enhanced growth characteristics

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/82, C12N15/52

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

Process for the production of plants with enhanced growth characteristics

1. Introduction

Nitrogen often is the rate-limiting element in plant growth. Most field crops have a fundamental dependence on inorganic nitrogenous fertilizer. Mineral fertilizers are a major source for ground water pollution. Therefore it would be beneficial if plants could utilize the existing nitrogen more efficiently.

Nitrogen is taken up by the plant as inorganic compounds, namely nitrate and ammonia. The majority of this nitrogen is assimilated into organic compounds like amino acids. The glutamine sythetase plays a major role since it catalyses the assimilation of ammonia into glutamine. Glutamine together with asparagines are the main transport forms of nitrogen in plants. As described in EP 511 979 the expression of an bacterial asparagines synthetases leads to improved growth characteristics which may be enhanced by the additional treatment of the plants with the herbicide glufosinate, a glutamine synthetases inhibitor. Whereas WO 95/09911 describes the production of a plant with improved agronomic or nutritional characteristics by over expression of one or several nitrogen/metabolism enzymes we were able to find a quite different way to improve plant growth characteristics.

2. Description of the invention

It has surprisingly be found that it is possible to improve plant growth capacities by the targeted expression of at least one bacterial asparagine synthetase in the chloroplast.

The present invention is directed to a process for the production f plants with improved growth characteristics which comprises the following steps:

- transfer and integration of a DNA sequence coding for a bacterial asparagin synthetases in the plant genome
- wherein said DNA sequence is linked to a regulatory sequence which ensures expression of said gene in a plant cell and leading to the import of the derived protein into the chloroplast and/or plastids of said plant cells and
- regeneration of intact and fertile plants from the transformed cells.

According to instant invention the term improved growth characteristics is to be understood as encompassing enhanced or faster and more vigorous growth as well as more yield and/or earlier flowering. The process according to instant invention leads also to bigger or more reproductive organs as for example the seeds.

According to instant invention the bacterial asparagines synthetases may also be expressed directly in the chloroplast by integrating the gene directly into the genome of the chloroplast and/or plastids by for example the biolistic transformation procedure (see US 5.451.513 incorporated herein by reference).

Therefore, the instant invention is also directed to a process for the production of plants with improved growth characteristics which comprises the following steps:

- transfer and integration of a DNA sequence coding for a bacterial asparagin synthetases into the genome of the chloroplast and/or plastids of a plant cells,
- expression of said gene under the control of appropriate regulatory elements and
- regeneration of intact and fertile plants from the transformed cells.

A preferred method of introducing the nucleic acid segments into plant cells is to

infect plant cells with A. tumefacient carrying an inserted DNA construct. The nucleic acid segments or constructs can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of A. tumefacient. The T-DNA is transmitted to plant cells upon infection by A. tumefacient, and is stably integrated into the plant genome. Under appropriate conditions known in the art, the transformed cells develop further into plants.

The Agrobacterium strains customarily employed in the art of transformation are described, for example see especially US 5.188.958 and EP 0 270 615 B1.

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumour formation. The other, termed virulent region, is essential for the introduction of the T DNA into plants. The transfer DNA region, which is transferred into the plant genome, can be increased in size by the insertion of the foreign nucleic acid sequence without its ability of transfer being affected. By removing the tumour-causing genes so that they no longer interfere the modified Ti plasmid ("disarmed Ti vector") can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate microspores. In the binary system, to have infection, two plasmids are needed: a T-DNA containing plasmid and a vir plasmid (see especially EP 116718 B1 and EP 120 516 B1).

Besides transformation using Agrobacteria there are many other techniques for the introduction of DNA available. These techniques include, e.g. the protoplast transformation (see EP 164 575) the micro injection of DNA, the introduction of DNA via electroporation as well as biolistic methods and virus mediated infection. From the transformed cells applying suitable media and techniques whole plants can be regenerated (see McCormick et al. (1986) in Plant Cell Reports 5: 81-84). The regenerated plants may be preferably used to cross them with existing breeding lines to improve their growth characteristics as well.

The DNA constructs used in instant invention consist of a transcription initiation region and, under the control of the transcription initiation region, a DNA sequence to be transcribed. The DNA sequence may comprise a natural open reading frame including transcribed 5' and 3' flanking sequences. Alternatively, it may comprise an anti-sense sequence that encodes the complement of an RNA molecule or portion thereof (as described in EP 140 308 B1 and EP 223 399 B1) in order to suppress the expression of the internally expressed glutamine synthetases.

The initiation regions may be used in a variety of contexts and in combination with a variety of sequences. The RNA coded sequences of a gene may be those of a natural gene, including the open reading frame for protein coding and frequently the 5' and 3' untranslated sequences. The RNA translational initiation sequences are included in the constructs, either from the promoter domain or from the attached coding sequences.

Attached to the above sequences are appropriate transcription termination and polyaderiylation sequences.

The DNA constructs used in the transformation process according to instant invention may comprise sequences coding for naturally occurring or genetically modified transit peptides (see for example EP 189 707 B1).

Examples of additionally expressed sequences or genes to be expressed from the constructs of the subject invention include:

- especially antisense or sense genes (for gene suppression or cosuppression); as well as additionally
- nutritionally important proteins: growth promoting factors;
- yield enhancing genes or factors, e.g. an invertase gene, a citrate synthase, a polyphosphate kinase;

- proteins giving protection to the plant under certain environmental conditions, e. g. proteins giving resistance to metal or other toxicity;
- stress related proteins giving tolerance to extremes of temperature, freezing, etc.
- proteins of specific commercial value;
- genes causing increased level of proteins, e. g., enzymes of metabolic pathways,
- genes causing increased levels of products of structural value to a plant host, e. g., herbicide resistance, fungus resistance, e.g. chitinase genes, glucanase genes, proteins synthesis inhibitor genes, ribosome inhibitory protein genes, viral resistance, e.g. ribozymes, virus coat protein genes.

The subject constructs will be prepared employing cloning vectors, where the sequences may be naturally occurring, mutated sequences, synthetic sequences, or combinations thereof. The cloning vectors are well known and comprise prokaryotic replication systems, markers for selection of transformed host cells, and restriction sites for insertion or substitution of sequences. For transcription and optimal expression, the DNA may be transformed into plant cells for integration into the genome, where the subject construct is joined to a marker for selection or is co-transformed with DNA encoding a marker for selection.

The selection of transformed cells is enabled by the use of a selectable marker gene which is also transferred. The expression of the marker gene confers a phenotypic trait that enables the selection. Examples for such genes are those coding for antibiotics or herbicide resistance, e.g. genes causing resistance against glutamine synthetases inhibitors, e.g. bialaphos or phosphinothricin resistance conferred by genes isolated from Streptomyces hygroscopicus or viridochromogenes (BAR/PAT). Other examples are the neomycin phosphotransferase or the glucuronidase gene.

The class of transgenic plants which are covered by this invention is generally as

broad as the class of higher plants susceptible to transformation, including both monocotyledonous and dicotyledonous plants. It is known that theoretically all plants can be regenerated from cultured totipotent cells, including but not limited to all major cereal crop species, sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables.

Examples of families that are of special interest are Poaceae, but also Solanaceae, Malvaceae and Brassicaceae.

Some suitable species include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, <<<MARK>>>
Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciohorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura.

Examples of species of commercial interest that can be protected include:

- tobacco, Nicotiana tabacum L.
- tomato, Lycopersicon esculentum Mill,
- potato, Solanum tuberosum L.,
- Canola/Rapeseed,
- Brassica napus L.,
- cabbage, broccoli, kale etc.,
- Brassica oleracea L.,
- mustards Brassica juncea L.,
- Brassica nigra L.,
- Sinapis alba L. (Brassicaceae),

- petunia, Petunia hybrida (Solanaceae)
- sugar beet, Beta vulgaris,(Chenopodiaceae),
- cucumber, Curcurbita sp. (Curcurbitaceae),
- cotton, Gossypium sp., (Malvaceae),
- sunflower, Helianthus annuus,
- lettuce Lactuca sativa, (Asteraceae = Compositae),
- pea, Pisum sativum,
- soybean, Glycine max and alfalfa, Medicago sp. (Fabaceae = Leguminoseae),
- asparagus, Asparagus officinalis;
- gladiolus, Gladiolus sp., (Liliaceae);
- corn, Zea mays;
- rice, Oryza sativa (Poaceae);
- wheat, Triticum aestivum (Poaceae); and
- barley, Hordeum vulgare (Poaceae).

In an preferred embodiment the invention covers transformed corn, sugar beet, cotton, rape seed, soy bean, lupine, rice and wheat.

The invention additionally relates to transformed plants which have been regenerated out of different cell types which have been transformed according to instant invention.

The transformation can be carried out as described in the materials and methods and in the examples.

In general, preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, Southern blots, DNA ligation and bacterial transformation were carried out using standard methods. (Maniatis et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1982), referred to herein as "Maniatis" and hereby incorporated by reference.)

3. Materials and Methods

The disclosure will then be completed with the description of the conditions under which the improved plant cells according to instant invention can be produced, also merely by way of examples for non-limitative illustration purposes.

3.1. Fusion of a bacterial asparagine synthetase gene to the nucleotide sequence for a duplicated chloroplast transit peptide

Based on the complete nucleotide sequence of the ASN-A gene from E. coli (Nakamura et al. (1981) or EP 511 979) the gene was cloned as a Hga 1 /Pst 1 fragment into the vector pUC18. By means of PCR based in vitro mutagenesis a SphI site was created at the ATG translational start codon changing the nucleotide sequence from AAA ATG AAA ACC GCT into GGC GCATG CAG AAA ACC GCT. This mutation introduced an additional codon for glutamic acid into the gene directly following the ATG translation start codon.

The nucleotide sequence for a modified transit peptide from the small subunit of Ribulosebisphosphat Carboxylase from pea was isolated from the vector pNi6/25 (Wasmann, C.C. et al (1986) Mol. Gen. Genet. 205: 446-453) as a Hind3/Sph1 fragment. This transit peptide contains a duplication of 20 amino acids compared to the natural transit

The sequence of the duplicated transit peptide and ASN-A gene were fused by ligating the Sph1 sites resulting in tpASN. The tpASN gene was exised as a Hind3/Pst1 fragment and after changing the Hind3 site into a Kpn1 site cloned between CaMV 35S promoter and -terminator of the vector pDH51 $^{\delta}$Kpn.

3.2. Expression of the tpASN gene in tobacco and rape seed

The 35S-promoter/tpASN gene/35S-terminator cassette from pDH51 $^{\delta}$Kpn was

isolated as an EcoR1 fragment, Hind3 linkers were added and the fragment was cloned into the Hind3 site of the vector pHOE6/Ac, which confers phosphinothricin resistance to plants. The resulting vector was called pHOE6Ac/tpASN This vector was transformed into the C58 Agrobacterium strain MP9ORK (Koncz et al. Mol. Gen. Gen., 204, 383-396 (1986)).

Tobacco and rape seed plants were transformed following published procedures. Plants were regenerated on Murashige and Skoog based media as described.

Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were analysed for the presence of the bacterial asparagine synthetase gene. In a Northern Blot analysis ASN-A specific RNA was detected in the plants. With polyclonal antibodies it could be demonstrated that the protein was targeted into the chloroplasts.

3.3. Expression of the tpASN gene in maize

The 35S-promoter/tpASN gene/35S-terminator cassette from pDH51 δKpn was isolated as an EcoR1 fragment, Hind3 linkers were added and the fragment was cloned into the Hind3 site of the vector pB2/35SAc resulting in pB35SAc/tpASN. This vector was used to transform maize protoplasts according to published procedures (EP 511 979 or Donn et al.). Plants were regenerated on Murashige and Skoog based media as described. Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were analysed for the presence of the bacterial asparagine synthetase gene. In a Northern Blot analysis ASN-A specific RNA was detected in the plants. With polyclonal antibodies it could be demonstrated that the protein was targeted into the chloroplasts.

3.4. Inhibition of chloroplastic glutamine synthetase by expression of the antisense gene in tobacco and rape seed

The coding sequences for the chloroplastic isoenzymes of Nicotiana sylvestris and Brassica napus were cloned by PCR methods from the genomic DNA of the respective plants. The resulting fragments were cloned as ApaI fragments in antisense orientation between 35S-promoter and -terminator from CaMV located on the vector pRT100. The 35S-promoter/GS-antisense/35S-terminator cassettes were isolated as Pst1 fragments and cloned into the Pst1 site of the vector pHOE6/AcK3. This vector was transformed into the C58 Agrobacterium strain MP9ORK (Koncz et al.). Tobacco and rape seed plants were transformed following published procedures. Plants were regenerated on Murashge and Skoog based media with reduced amounts of ammonia as described.

Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT resistant plants were screened with Southern Blot hybridization for the presence of the ASN-A gene. Southern positive plants were analysed for the inactivation of the chloroplastic glutamine synthetase gene by Northern blots. Plants with the most reduced GS RNA level were selected.

3.5. Inhibition of chloroplastic glutamine synthetase by expression of the respective antisense gene in maize

The coding sequences for the chloroplastic isoenzymes of Zea mays, was cloned by PCR methods from the genomic DNA. The resulting fragment was cloned as ApaI fragment in antisense orientation between 35S-promoter and terminator from CaMV located on the vector pRT100. The 35S-promoter/GS-antisense/35S-terminator cassette was isolated as Pst1 fragment and cloned into the vector pB2/AcK3.

This vector was used to transform maize protoplasts according to published procedures (Donn et al.). Plants were regenerated on Murashge and Skoog based media with reduced amounts of ammonia as described. Transformed plants were selected because of their resistance to the herbicide phosphinothricin (PPT). PPT

resistant plants were screened with Southern Blot hybridization for the presence of the ASN-A gene. Southern positive plants were analysed for the inactivation of the chloroplastic glutamine synthetase gene by Northern blots. Plants with the most reduced GS RNA level were selected.

3.6. Asparagin content in transgenic asparagin synthetase expressing plants

Leaf material from wild type and different ransgenic asparagin synthetase expressing plants was homogenized in buffer. The extracts were run over a Biotronic amino acid analyser. Concentration of the amino acid asparagine were measured and are given in pmol/µl of extract.

	NT-WT	NT-TPASN-2	NT-TPASN-3	NT-TPASN-5	NT-TPASN-11
ASN	586,855	890,26	3338,5551	1506,6314	992,0319

The concentration of asparagine correlated with the expression of the asparagine synthetase gene as measured on Northern and Western Blots.

3.7. Growth behaviour of transgenic maize and tobacco plants

Transgenic asparagin synthetase expressing plants and transgenic asparagin synthetase expressing plants with reduced glutamine synthetase activity were grown side by side with wild type plants in the greenhouse. The transgenic plants showed a more vigorous growth and flowered earlier than wild type plants.

Claims:

1. A process for the production of plants with improved growth characteristics which comprises the following steps:
   - transfer and integration of a DNA sequence coding for a bacterial asparagin synthetase in the plant genome
   - wherein said DNA sequence is linked to a regulatory sequence which ensures expression of said gene in a plant cell and leading to the import of the derived protein into the chloroplasts and/or plastids of said plant cells and
   - regeneration of intact and fertile plants from the transformed cells.

2. A plant cell expressing a prokaryotic ammonium specific asparagine synthetase in its chloroplasts and plastids.

3. A plant cell according to claim 2 expressing further a gene construct leading to reduced level of its endogenous glutamine synthetase activity.

4. A plant, seeds and propagation material containing cells as claimed in claims 2 and 3.

5. A gene construct comprising a gene encoding a prokaryotic ammonium specific asparagine synthetase operatively linked to a regulatory sequence which ensures expression of said gene in a plant cell and leading to the import of the derived protein into chloroplasts and/or plastids of said plant cell.

6. A gene construct according to claim 4, wherein the asparagine synthetase gene is an E.coli asparagine Synthetase gene with a chloroplastic leader peptide at its N-terminus.

7. A vector containing a gene construct according to claims 5 and 6.

8. A plant cell transformed with the gene construct according to claim 5 and 6 or with a vector according to claim 7.

Abstract: AGR 96/M 206

Process for the production of plants with enhanced growth characteristics

Process for the production of plants with improved growth characteristics by targeted expression of an bacterial asparagines synthetase in the chloroplasts or plastides.